Roles of NO and oxygen radicals in tubuloglomerular feedback in SHR

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Welch, William J., Akihiro Tojo, and Christopher S. Wilcox. Roles of NO and oxygen radicals in tubuloglomerular feedback in SHR. Am J Physiol Renal Physiol 278: F769–F776, 2000.—The spontaneously hypertensive rat (SHR) has enhanced tubuloglomerular feedback (TGF) responses and diminished buffering by juxtaglomerular apparatus (JGA)-derived nitric oxide (NO) despite enhanced expression of NO synthase (NOS) isoforms in the JGA. We tested the hypothesis that the enhanced TGF response is due to inactivation of NO by oxygen radicals (O2·). SHR had significantly (P < 0.05) greater expression of the peroxynitrate reaction product, nitrotyrosine, in renal cortex. A membrane-permeant, metal-independent superoxide dismutase mimetic, tempol, was used to test the functional role of O2·. Maximum TGF responses, assessed from changes in proximal stop-flow pressure (Psf) during orthograde loop of Henle (LH) perfusion of artificial tubular fluid (ATF), were enhanced in SHR [Wistar-Kyoto rat (WKY) 8.8 ± 0.4 (n = 30 nephrons) vs. SHR 10.8 ± 0.4 mmHg (n = 39 nephrons), P < 0.001]. TGF responses of SHR were unresponsive to microperfusion of 7-nitroindazole (7-NI, 10–4 M), which is an inhibitor of neuronal NOS (nNOS) mRNA and protein expression for nNOS and eNOS in nephrons of SHR is presently unexplained. The overactive TGF in the genetic SHR model of hypertension has been ascribed to a diminished role for NO from nNOS in macula densa cells (39). The overactive TGF in the genetic SHR model of hypertension has been ascribed to a diminished role for macula densa NO in buffering the vasoconstrictive response (34, 36). Thus SHR has a diminished or absent TGF response to local inhibition of NO in the juxtaglomerular apparatus (JGA) with nitro-L-arginine (NLA) (34) or nNOS with 7-nitroindazole (7-NI) (36).

The cause for the diminished blunting of TGF by NO in nephrons of SHR is presently unexplained. The mRNA and protein expression for nNOS and eNOS in the JGA of SHR is enhanced compared with control Wistar-Kyoto rats (WKY) (36). TGF is unresponsive to microperfusion of the NOS substrate L-arginine or the NOS cofactor tetrahydrobiopterin or its precursor sepiapterin into the macula densa segment (36). Therefore, there does not appear to be a defect in the expression of constitutive NOS isoforms, or in the delivery of NOS substrate or cofactors to the JGA to explain the defective function of NO in the SHR.

The aorta of SHR has an impaired endothelium-derived relaxing factor (EDRF) response. This is ascribed to an endothelium-derived contraction factor (EDCF) that can inactivate NO (18). The precise identity of EDCF remains unclear, but its generation or action depends on oxygen radicals (O2·). O2· interacts with NO to yield peroxynitrate (ONOO−), which effectively reduces the biological half-life of NO. Peroxynitrate itself can interact with tyrosine residues to form

NITRIC OXIDE (NO) is implicated in the regulation of renal vascular resistance, glomerular filtration, tubular reabsorption, renin secretion, and tubuloglomerular feedback (TGF) responses (15, 36–38). TGF is a vasoconstrictive response of the afferent arteriole that reduces the glomerular capillary pressure and the single-nephron glomerular filtration rate (SNGFR) during reabsorption of NaCl by the macula densa segment (7). This vasoconstrictive response is enhanced in the spontaneously hypertensive rat (SHR) model of hypertension and may contribute to renal mechanisms for the development of hypertension (9). A neuronal or type I isoform of nitric oxide synthase (nNOS) is heavily expressed in macula densa cells (35, 39) and an endothelial or type III isoform of NOS (eNOS) is expressed in microvascular and glomerular capillary endothelium (33, 35). Pharmacological studies have shown that nNOS in macula densa cells is activated during NaCl reabsorption. The ensuing generation of NO offsets the primary vasoconstrictive effects of TGF on the afferent arteriole and diminishes further NaCl reabsorption by the macula densa cells (39). The overactive TGF in the genetic SHR model of hypertension has been ascribed to a diminished role for macula densa NO in buffering the vasoconstrictive response (34, 36). Thus SHR has a diminished or absent TGF response to local inhibition of NO in the juxtaglomerular apparatus (JGA) with nitro-L-arginine (NLA) (34) or nNOS with 7-nitroindazole (7-NI) (36).

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nitrotyrosine. In the 2-kidney, 1-clipped rat model of hypertension, nitrotyrosine immunoreactive expression is prominent in the extraglomerular mesangium and interstitium between the macula densa cells and the afferent arteriole (5). Therefore, the JGA appears to be a prominent site for generation of $O_2^-$ that could inactivate NO formed in the macula densa.

We tested the hypothesis that a defect in NO function in the JGA of the SHR despite ongoing NO generation could be ascribed to enhanced degradation of NO via interaction with $O_2^-$. Nitrotyrosine immunoreactivity was used to assess the net interaction between renal NO and $O_2^-$. TGF responses to microperfusion of 7-NI were used to assess the role of nNOS in the macula densa. The response to NO was assessed by microperfusion of $S$-nitroso-$N$-acetyl-penicillamine (SNAP), which is an NO donor compound. The nitroxide, tempol (2,2,6,6-tetramethyl-1-piperidinyloxy, 8-hydroxy form), is a low-molecular-weight membrane-permeant, metal-independent, stable, superoxide dismutase (SOD) mimetic that is used as an $O_2^-$ spin trap. Tempol was microperfused into the vasculature surrounding the JGA to assess the role of $O_2^-$. Series 2: Maximal TGF responses to luminal microperfusion of the nNOS inhibitor, 7-NI, and effects of efferent arteriolar microperfusion of tempol. These experiments were designed to test the hypothesis that the enhanced TGF responses of SHR nephrons could be ascribed to a blunted generation of NO by nNOS in the macula densa because of enhanced degradation by $O_2^-$. Groups of SHR and age-matched WKY rats were prepared for in vivo micropuncture, microperfusion, and TGF studies as described in detail previously. In brief, animals weighing 210–275 g were anesthetized with thiobarbital (Inactin, 100 mg/kg ip; Research Biochemicals International, Natick, MA). A catheter was placed in a jugular vein for fluid infusion and in a femoral artery for recording of mean arterial pressure (MAP) from the electrically damped output of a pressure transducer (Statham). A tracheotomy tube was inserted and the animals were

**Fig. 1. Representative Western blot for nitrotyrosine in renal cortex homogenate. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat.**

**Fig. 2. Density of nitrotyrosine immunoreactivity (means ± SE) by Western analysis of protein extracted from kidneys of WKY (n = 7) and SHR (n = 7).**
allowed to breathe spontaneously. The left kidney was exposed by a flank incision, cleaned of connective tissue, and stabilized in a Lucite cup. This kidney was bathed in 0.154 M NaCl maintained at 37°C. After completion of surgery, rats were infused with a solution of 0.154 M NaCl and 1% albumin at 1.5 ml/h to maintain a euolemic state. Micropuncture studies were begun after 60 min for stabilization.

For orthograde microperfusion of the loop of Henle (LH), a micropipette (8 µm OD) containing artificial tubular fluid (ATF) stained with FD&C green dye was inserted into a lateral proximal tubule. Injections of the colored ATF identified the nephron and the direction of flow. An immobile stained bone wax block was inserted into this micropuncture site via a wax block to connect to a hydraulic drive (Trent Wells, San Diego, CA) to halt tubular fluid flow. A perfusion micropipette (10–15 µm) connected to a hydraulic drive was inserted into the EA adjacent to the test nephron. The LH was perfused orthogradely with ATF at zero and 40 nl/min. This represents the maximal TGF response. Preliminary studies indicated that a dose of tempol of 10⁻⁴ M was maximally effective, and the response was fully reversible. Therefore, this dose was used for these studies. After the basal measurement of TGF with LH perfusion of ATF, the EA was perfused at 15 nl/min with AP + tempol. After 5 min with the EA microperfusion continuing, the TGF was again tested by perfusion of ATF at 0 and 40 nl/min into the LH. We have shown that PTC perfusion with AP at 15–20 nl/min does not perturb TGF responses. Only nephrons for which the surrounding interstitium was tinted with the PTC perfusion of stained tempol solution were included in the study.

A further group of six WKY and nine SHR were used to test the hypothesis that the failure of TGF responses of SHR nephrons to enhance with local microperfusion of 7-NI is due to degradation of NO by O₂ in the JGA. The maximum TGF responses of SHR and WKY to orthograde microperfusion of ATF + vehicle were compared with ATF + 7-NI (10⁻⁴ M) during a 5-min microperfusion of tempol (10⁻⁴ M) in AP at 15 nl/min into the EA.

Series 3: Dose-response relationship of TGF to microperfusion of NO donor compound into JGA: effects of tempol. The aim of this series was to test the hypothesis that graded doses of NO, delivered directly into the JGA, have a reduced biologic activity in SHR nephrons that can be corrected by a membrane-permeant SOD mimetic. Rats were prepared as in series 2 above. The maximum TGF responses of SHR (n = 16 nephrons) and WKY (n = 18 nephrons) to orthograde microperfusion of ATF + SNAP (10⁻⁷ M, 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M) were studied in the basal state, and during EA microperfusion of tempol (10⁻⁴ M).

Drugs. The soluble sodium salt of 7-NI was obtained from Tocris-Cookson, St. Louis, MO. Tempol was obtained from Aldrich Chemical, Milwaukee, WI. SNAP was obtained from Sigma, St. Louis, MO. These drugs were dissolved in artificial plasma (AP) at 30°C.

Statistical methods. Values are reported as means ± SE. An ANOVA was applied to within-group data for SHR and WKY; where appropriate, a post hoc Dunnett’s t-test was

### Table 1. Basal values for animals studied in series 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of Rats</th>
<th>No. of Nephrons</th>
<th>Wt. g</th>
<th>MAP, mmHg</th>
<th>GFR, ml·min⁻¹·100 g⁻¹</th>
<th>HR, beats/min</th>
<th>Pₛᵢ (mmHg) During LH Perfusion at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>16</td>
<td>30</td>
<td>242±10</td>
<td>117±6</td>
<td>0.81±0.08</td>
<td>344±10</td>
<td>36.9±0.6</td>
</tr>
<tr>
<td>SHP</td>
<td>19</td>
<td>39</td>
<td>254±7</td>
<td>152±6</td>
<td>0.61±0.07</td>
<td>372±12</td>
<td>39.8±0.5</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE. SHR, spontaneously hypertensive rat; WKY, Wistar-Kyoto rat; MAP, mean arterial pressure; GFR, glomerular filtration rate; HR, heart rate; Pₛᵢ, stop-flow pressure; LH, loop of Henle; NS, not significant.

### Table 2. TGF response to orthograde loop microperfusion of 7-NI

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of Rats</th>
<th>No. of Nephrons</th>
<th>Loop Microperfusion</th>
<th>Pₛᵢ (mmHg) During LH Perfusion at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>5</td>
<td>8</td>
<td>ATF + Veh</td>
<td>36.7±0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATF + 7-NI</td>
<td>37.0±0.7</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>NS</td>
<td>10.8±0.4</td>
</tr>
<tr>
<td>SHR</td>
<td>5</td>
<td>8</td>
<td>ATF + Veh</td>
<td>38.8±0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATF + 7-NI</td>
<td>39.0±0.8</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>NS</td>
<td>10.0±0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. TGF, tubuloglomerular feedback; ATF, artificial tubular fluid; Veh, vehicle; 7-NI, 7-nitroindazole (10⁻⁴ M).
applied thereafter. Values are taken as statistically significant at \( P < 0.05 \).

Numerical data for TGF responses are presented in Tables 1-4. However, the basal TGF responses were greater in SHR than WKY. Therefore, to adjust for baseline differences, the results are shown graphically as percentage changes in TGF from baseline values, according to the criteria of Kaiser (13).

RESULTS

For series 1, immunoreactive bands corresponding to nitrotyrosine were seen at 47, 58, 74, 89, and 102 kDa in proteins extracted from rat renal cortex. These bands were consistently stronger in kidneys from SHR than WKY (Fig. 1). Densitometric analysis of bands at 58 kDa confirmed that the amount of nitrotyrosine immunoreactive protein expression was significantly greater in SHR than WKY kidneys (Fig. 2).

Table 1 presents baseline data for rats used in the functional studies of series 2. Compared with WKY, SHR had similar body and kidney weights but had consistently higher levels of MAP and slightly greater heart rates (HR). TGF parameters showed higher values for \( P_{sf} \) in SHR nephrons in the absence of loop perfusion and a greater maximal TGF response, as assessed from the difference between \( P_{sf} \) during perfusion at 0 and 40 nl/min. The maximum response in SHR averaged 123 ± 10% of the mean value in WKY.

The maximum TGF responses were contrasted in SHR and WKY nephrons during microperfusion of 7-NI orthograde into the LH. As shown in Table 2, the maximum TGF responses again were greater in SHR than WKY during perfusion of ATF vehicle. The addition of 7-NI increased maximal TGF responses consistently in WKY by an average of 30 ± 4% but had no significant effects on TGF responses of SHR (Fig. 3A).

Microperfusion of tempol into the EA blunted maximum TGF responses significantly (\( P < 0.01 \)) in both WKY and SHR nephrons (Table 3). However, as shown in Fig. 3B, the percentage of blunting of TGF in nephrons of SHR was nearly double that in WKY.

During microperfusion of tempol into the EA, orthograde loop perfusion of 7-NI increased TGF responses in WKY rat nephrons by 28 ± 5%; this is similar to the 30 ± 4% increase seen with 7-NI in WKY in the absence of tempol (Table 2). However, during EA microperfusion of tempol in SHR nephron, orthograde loop perfusion of 7-NI increased TGF by 32 ± 5% (Fig. 3C). This is significantly (\( P < 0.001 \)) more than the 5 ± 3% (not significant) change seen with 7-NI in SHR in the absence of tempol. During tempol, the changes in maximal TGF with 7-NI become quite similar in WKY and SHR (Fig. 3C; Table 4).

For series 3, log-dose response relationships for blunting of maximal TGF responses during orthograde luminal microperusions of SNAP into the LH are shown in the basal state (Fig. 4A) or during EA microperfusion of tempol (Fig. 4B). Before tempol, SNAP at \( 10^{-7} \) M was a subthreshold dose in both WKY and SHR, as indicated by nonsignificant changes in TGF responses. SNAP at \( 10^{-8} \) M blunted TGF significantly (\( P < 0.05 \)) in WKY but not in SHR nephrons. SNAP at \( 10^{-5} \) M blunted TGF significantly (\( P < 0.05 \)) more in WKY than in SHR nephrons. The responses to a full dose of SNAP at \( 10^{-4} \) M were similar in the two strains. As shown in Fig.
Table 4. TGF response to orthograde loop microperfusion of 7-NI during EA microperfusion of tempol

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of Rats</th>
<th>No. of Nephrons</th>
<th>Loop Microperfusion</th>
<th>P_{tr} (mmHg) During LH Perfusion at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0 nl/min</td>
</tr>
<tr>
<td>WKY</td>
<td>6</td>
<td>12</td>
<td>ATF + Veh</td>
<td>36.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATF + 7-NI</td>
<td>36.4 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>SHR</td>
<td>9</td>
<td>16</td>
<td>ATF + Veh</td>
<td>39.1 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ATF + 7-NI</td>
<td>39.5 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE.
dance of mRNA and protein for eNOS and nNOS in the JGA or renal cortex are actually enhanced in SHR, relative to WKY (36). Nor can it be due to a failure to supply the NOS substrate, L-arginine, or the NOS cofactor, tetrahydrobiopterin, to the JGA, since microperfusion of these agents into the macula densa does not modify TGF, or its response to 7-NI, in the SHR (36).

Therefore, the present studies were undertaken to assess a contrasting hypothesis that defective NO function in the JGA of the SHR is due rather to a failure to respond to NO, because of enhanced degradation. It has long been known that the short biological half-life and restricted diffusion distance of NO in blood vessel walls are due to rapid and irreversible inactivation by oxygen radicals that oxidize NO initially to peroxynitrite (11, 27). Peroxynitrite can nitrosylate tyrosine epitopes leading to nitrotyrosine formation whose expression provides an index of oxidative stress (32). A consistent and strong band of immunoreactive nitrotyrosine was detected in proteins extracted from the renal cortex. The nature of this predominant nitrated tyrosine antigen is unclear. Nevertheless, its expression was enhanced consistently in the renal cortex of SHR, compared with WKY. This finding extends our previous observation that isoprostane excretion is increased in SHR and that this increase can be normalized by a 2-wk administration of tempol (30). Isoprostanes are formed by nonenzymic interactions between O$_2$ and arachidonate. These findings at the level of the kidney and whole circulation complement extensive previous studies of isolated vessels of SHR that have shown an enhanced agonist-induced release of EDCF that accounts for a diminished EDRF activity (3). EDCF depends upon oxygen radicals (1), cyclooxygenase (17), and thromboxane-PGH$_2$ (TP) receptors (2). In a recent study, Brannstrom et al. (6) have shown that much of the exaggerated TGF response in SHR is dependent on activation of TP receptors. This may relate to increased O$_2$ activity, through formation of isoprostanes that act on TP receptors (26). An interaction of O$_2$ with both NO and arachidonate could represent an additional level of regulation of TGF during oxidant stress.

Previous studies of EDCF and O$_2$ have shown that they are important mediators of large vessel responses in hypertension and atherosclerosis (1). The present studies are the first to demonstrate a role for O$_2$ in the JGA in regulation of afferent arteriolar tone via interaction with NO.

Evidence that oxidant stress and O$_2$ account for exaggerated preglomerular vasoconstriction, enhanced TGF, and a diminished functional role for NO in the JGA of the SHR were derived from two sets of experiments.

First, the blunting of TGF by the NO donor compound, SNAP, showed a diminished sensitivity but a preserved maximal responsiveness. The preserved responsiveness suggests that NO responsive elements in the SHR afferent arteriole were intact. The diminished sensitivity to SNAP in SHR is consistent with enhanced NO degradation via O$_2$. This conclusion was strengthened by the observation that microperfusion of tempol via the EA normalized the sensitivity to SNAP in the SHR. Our interpretation is that the SOD mimic, tempol, metabolizes O$_2$ to H$_2$O$_2$ and O$_2$, which do not directly interact with NO. Therefore, tempol enhances the effectiveness of physiologically relevant concentrations of NO by diminishing its rapid degradation. Since this effect of tempol is more pronounced in SHR than WKY, it implies that O$_2$-induced NO degradation similarly is more pronounced in SHR than WKY nephrons.

Second, we observed that nephrons of SHR were approximately twice as responsive as WKY to microperfusion of tempol, as shown by twice as great a fractional blunting of TGF in the hypertensive model (Fig. 3B). If this effect of tempol is due to activation of a latent NO-dependent vasodilator mechanism that offsets TGF
responses, we argued that tempol should restore a normal response to nNOS inhibition in the SHR. Indeed, microperfusion of tempol into the EA normalized the response to microperfusion of 7-NI into the macula densa of the SHR (Fig. 3C). We conclude that the failure of the JGA L-arginine-NO pathway to function in the SHR can be ascribed to excessive NO degradation by O$_2$-$^*$, rather than to an intrinsic failure in the system to generate NO in the macula densa or to respond to NO in the vessel wall.

The present studies leave unanswered the source of O$_2$ generated in the JGA. Nitrotyrosine immunoreactivity in the 2-kidney, 1-clipped Goldblatt hypertensive rat model is expressed densely in the interstitium and the extraglomerular mesangial cells between the macula densa and the afferent arteriole (5). The interstitium and extraglomerular mesangium may form a barrier that isolates NO generated in the macula densa from targets in the afferent arteriole. Mesangial cell NADPH oxidase is stimulated to generate O$_2$ by ANG II acting on AT$_1$ receptors (12). Therefore, NADPH oxidase could be the predominant source of O$_2$ generation in the JGA.

Since this source of O$_2$ is strongly dependent on AT$_1$ receptor activity, the level of oxidative stress in the SHR may vary with age. We have tested 9- to 10-wk-old rats. This is a time when hypertension is fully achieved and an exaggerated TGF is present. Although enhanced TGF is more often seen in young SHR (6, 9, 11), decreased renal blood flow and GFR have been observed in mature SHR (10, 14, 34). The level of ANG II also varies in the SHR. Most studies agree, however, that blockade of ANG II with either angiotensin converting enzyme inhibitors or AT$_1$ receptor antagonists reduces MAP in the SHR, regardless of age (reviewed in Ref. 16). Therefore, the effectiveness of NO in regulating TGF may well depend on the physiological state of the animal, which includes the level of oxidative stress and ANG II activity.

Previous studies have shown the importance of O$_2$ in signaling ANG II actions in blood vessel wall (25). ANG II concentrations probably are higher in the JGA than anywhere else in the body. The exaggerated TGF response of the SHR kidney is dependent on AT$_1$ receptors (6). Therefore, the JGA of the SHR should be a prominent site for ANG II-induced O$_2$ generation. An overactive TGF mechanism would contribute to inappropriate preglomerular renal vasoconstriction and a limited capacity to excrete a salt load rapidly and effectively. As such, it may be an important pro-hypertensive mechanism. Indeed, Ollerstam et al. (23) have shown that inhibition of nNOS with 7-NI first increases TGF responsiveness and reduces renal function of normal rats. Later, 7-NI increases the blood pressure. The present studies have shown that one factor responsible for an exaggerated TGF response in SHR is overproduction of O$_2$ leading to a functional state of NO deficiency. That this could contribute to maintenance of hypertensive responses is shown by the normalization of renal vasoconstriction and hypertension by short-term infusion of tempol (31) and by the selective reduction in blood pressure in the SHR by 2 wk of oral tempol administration (30).

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